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Molecular mechanisms underlying the antimetastatic activity of bufalin (Review)

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Abstract. Bufalin is a monomer compound extract from Chansu, which is a traditional Chinese medicine obtained from the skin and parotid venom glands of toads, such as Bufo bufo gargarizans Cantor and Bufo melanostictus Schneider. Chansu had been used in traditional Chinese medicine for >1,000 years due to its cardiac, anti-inflammatory and anti-cancer properties. Previous studies identified bufalin as the main anticancer compound of Chansu, and recent evidence has corroborated its anticancer properties. Bufalin inhibits cancer cell proliferation, induces cell cycle arrest, induces cancer cell apoptosis, inhibits neovascularization, induces cell differentiation, inhibits cancer metastasis and invasion, and enhances chemotherapeutic drug sensitivity. However, the function and mechanism of bufalin in metastatic cancer cells have not yet been expounded. The aim of the present review was to discuss the recent progress and prospects of bufalin in the prevention of cancer metastasis, particularly in inhibiting epithelial-to-mesenchymal transition.

Contents

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1. Introduction

Cancer represents a major health concern worldwide. Although the mortality rate of malignant tumours has been controlled due to the scientific and technological advances in the field of oncology, it remains one of the deadliest diseases (1). The majority of cancer-related deaths may be attributed to the metastatic spread and invasion of cancer cells into other vital organs. During this process, cancer cells degrade the basement membrane and extracellular matrix (ECM) and migrate to adjacent areas, where they then invade the blood and/or lymphatic vessels and reach other organs or tissues via the circulation. Growth factors, ECM proteins, intercellular adhesions, the cytoskeleton and genes all participate in this process (2). Therefore, it is of great significance to further elucidate the molecular mechanisms underlying cancer development and design effective antitumour drugs to improve prevention and therapeutic strategies against cancer.

Epithelial-to-mesenchymal transition (EMT) is a process by which cells lose their epithelial characteristics and acquire a mesenchymal cell phenotype. EMT is important in embryonic development, chronic inflammation, tissue reconstruction, a variety of fibrotic diseases and cancer metastasis (3,4). During the process of EMT, the cell-to-cell and cell-to-matrix connections become weaker and the epithelial polarity acquires mesenchymal characteristics, promoting cancer cell migration and invasion of the surrounding matrix or other organs (5-7). Recently accumulated evidence indicates that EMT contributes to lymph node metastasis, distant metastasis, prognosis and chemoresistance of cancers (8,9).

During EMT, the expression of epithelial cell markers, such as E-cadherin, is downregulated, while mesenchymal cell markers, such as vimentin and N-cadherin, are upregulated. Transcription factors, such as Snail, Zeb and Twist, are also involved in these processes. It was also revealed that certain signalling pathways are involved in EMT, such as the transforming growth factor (TGF)-β signalling pathway (10), Wnt signalling pathway (11), Hedgehog signalling pathway (12), and Notch signalling pathway (13). The development of drugs or interventions to reverse the process of EMT has become an important target of cancer research (14,15).

2. Bufalin suppresses cancer metastasis by inhibiting EMT

Bufalin is a bioactive polyhydroxy steroid isolated from Venenum Bufonis, also referred to as Chansu, a well-known

traditional Chinese herb. Chansu has been widely used in the clinical treatment of several malignancies in China (16,17). Recent studies demonstrated that bufalin exhibits anticancer activity against various cancers, such as breast cancer (18), osteosarcoma (19), colon cancer (20), lung cancer (21), pancreatic cancer (22), bladder cancer (23) and hepatocellular carcinoma (24). The underlying mechanisms include inhibiting cell proliferation (19), promoting cell apoptosis and autophagy (25,26), blocking the cell cycle (27), reversing multidrug resistance (28), and inhibiting EMT and cancer metastasis (24). Bufalin has been shown to affect several EMT signalling pathways.

3. Bufalin affects EMT by inhibiting TGF-β

The TGF-β family is a superfamily that regulates cell proliferation, apoptosis, the cell cycle, ECM transformation and tumour metastasis (29). The TGF-β family includes three isoform ligands, namely TGF-β1, TGF-β2 and TGF-β3, and two receptors, TβRI and TβRII. When the TGF-β ligand binds to the receptor, it leads to the phosphorylation of SMAD2 and SMAD3. Subsequently, SMAD4 is translocated into the nucleus and leads to the activation of target genes (30). Recent studies have found that the activation of TGF-β is involved in the EMT process and increases the potential of cancer metastasis. The EMT transcription factors targeted by TGF-β include Snail, two-handed zinc finger factors ZEB1 and ZEB2, and the basic helix-loop-helix Twist1 and Twist2 (31-33).

It was reported that bufalin can inhibit EMT in cancer cells via the TGF-β pathway. Zhao *et al* (34) reported that bufalin downregulates the expression of TGF-β receptors and inhibits TGF-β-induced EMT and the migration of A549 lung cancer cells. That study demonstrated that, following treatment with TGF-β, the migratory ability of lung cancer cells increased, and the cell morphology resembled that of mesenchymal cells. When treated with bufalin, these phenomena were markedly suppressed. The expression of Twist2, ZEB2, SMAD2 and SMAD3 was also downregulated following treatment with bufalin. Further analysis revealed that the TGF-β receptors TβRI and TβRII were also downregulated in the presence of bufalin. When the phosphorylation of TβRI was blocked by the inhibitor SB431542, the TGF-β-induced EMT was attenuated, similar to treatment with bufalin. It was also demonstrated that bufalin inhibited the migration and invasion of human colon cancer HCT116 cells and increased the ability of cell adhesion. Following treatment with bufalin, the expression of TGF-β1, SMAD4 and E-cadherin increased, while the expression of phospho-SMAD3 decreased. These proteins may be associated with bufalin and can induce the nuclear translocation of β-catenin and prevent cancer cell EMT (35,36).

4. Bufalin affects EMT through the PI3K/AKT signalling pathway

The phosphoinositide 3-kinase (PI3K)/AKT signalling pathway is involved in cell proliferation, cell cycle regulation and cell apoptosis. In addition, abnormal activation of AKT is associated with tumour occurrence and development. Overexpression of PI3K/AKT inhibits the apoptosis of cancer cells, and some chemotherapeutic drugs induce cell apoptosis by inhibiting the activation of AKT (37,38). It was also demonstrated that activated PI3K/AKT cell signalling may promote the degradation of ECM and reduce cell-cell adhesions, inducing EMT and thereby promoting the metastasis and invasion of malignant tumour cells (39). The overexpression of AKT promotes the expression of Twist and Snail, which are important in the activation of EMT. Treatment with an AKT inhibitor can restore the epithelial cell morphology to a polygonal shape and suppress the migration ability of cancer cells. While the expression of Twist and Snail was downregulated, E-cadherin expression was upregulated (40).

Wang *et al* (24) reported that bufalin suppresses liver cancer invasion and metastasis by downregulating the expression of the PI3K/AKT/mammalian target of rapamycin (mTOR) signalling pathway. That study demonstrated that, after treatment with bufalin, the liver/lung metastases were significantly reduced in mice with transplanted tumours. Furthermore, EMT was inhibited by bufalin, the expression of E-cadherin was upregulated, and the expression of N-cadherin, vimentin and Snail was downregulated. Moreover, the expression of TGF-β1 was inhibited after treatment with bufalin. It was also observed that bufalin can inhibit the expression of hypoxia-inducible factor-1α (HIF-1α) by suppressing the activation of the PI3K/AKT/mTOR pathway (41). Another study reported that bufalin can prevent hepatocellular carcinoma invasion and metastasis. Functional studies demonstrated that bufalin can inhibit matrix metalloproteinase (MMP)2 and MMP9 expression, as well as the expression of PI3K and the phosphorylation of AKT, thereby attenuating the expression of nuclear factor (NF)-κB (42).

Bufalin was also found to inhibit endometrial carcinoma cell migration and invasion. The expression of MMP9 and Snail was downregulated following treatment with bufalin, while the expression of E-cadherin was upregulated. Functional studies demonstrated that bufalin inhibits cervical tumourigenesis and metastasis by modulating α2/β5/FAK cell signalling and affecting the expression of PI3K/AKT. Integrins α2/β5 and downstream related genes FAK, pFAK, pGSK3β, AKT1 and p-AKT1 were all downregulated following treatment with bufalin (43).

5. Bufalin affects EMT through the Wnt/β-catenin signalling pathway

Wnt signalling comprises a group of signal transduction pathways consisting of proteins that transmit signals into the cell through cell surface receptors. There are three characterized Wnt signalling pathways: The canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway (44). The Wnt/β-catenin pathway is crucial for cellular maintenance and development, such as cell cycle progression, apoptosis, differentiation, migration and proliferation (45). The overstimulation of these pathways is closely associated with cancer development. During the development of cancer, the phosphorylation and/or nuclear localization of β-catenin increases, and the Wnt/β-catenin pathway is activated, increasing the migration and invasion ability of cancer cells (46). Furthermore, the Wnt/β-catenin pathway induces EMT by activating Twist1, ZEB1, Snail and Slug (47).

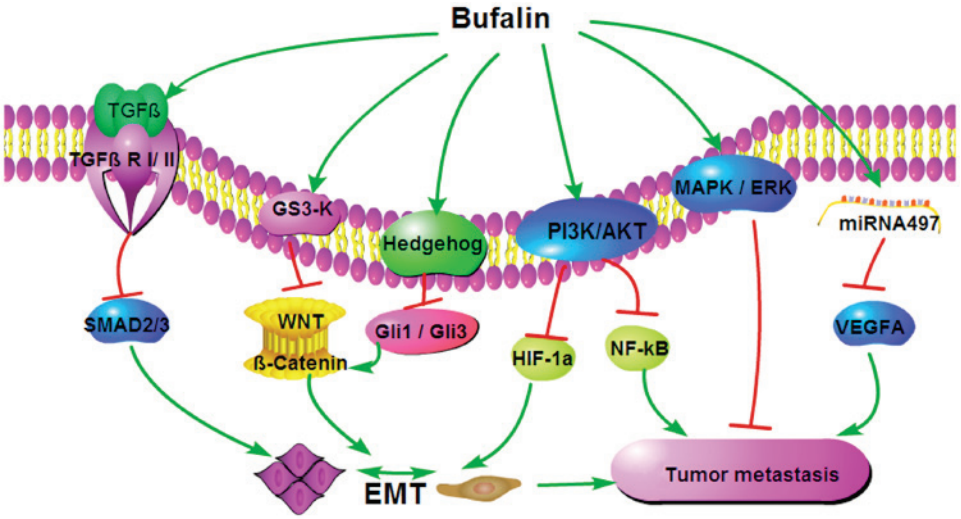

Figure 1. Pathways related to the anticancer activity of bufalin.

Gai *et al* (48) observed that bufalin inhibited the proliferation, migration and invasion of the hepatocellular carcinoma cell line BEL-7402. Upon investigating the underlying mechanism, they found that bufalin suppressed the phosphorylation of the GSK-3β Ser9 site in BEL-7402 cells and decreased the expression of cyclin D1, MMP7 and cyclooxygenase-2. They also found that bufalin inhibited the transfer of β-catenin to the nucleus, which is a key step in the Wnt/β-catenin signalling pathway.

6. Bufalin inhibits EMT through the Hedgehog signalling pathway

The Hedgehog (Hh) signalling pathway is highly conserved and is essential for the development of the normal embryo, particularly during early embryogenesis and morphogenesis of specific organs and tissues (49). The Hh pathway is usually silenced in most adult tissues; however, after injury, the pathway is reactivated to promote the repair and regeneration of cells. Furthermore, aberrant Hh signalling has been detected in a number of human cancers and has been implicated in tumourigenesis (50,51). When Hh signalling is silenced, the receptor Patched (PTCH1) binds the receptor Smoothened (SMO), inhibiting SMO and its downstream pathway activity. When PTCH1 binds its ligand, Sonic Hedgehog (SHH), SMO is activated and, subsequently, the glioma-associated oncogene Gli transcription factor is activated (52). Studies have found that Gli family members are associated with cancer cell EMT and metastasis, and Gli is also associated with the Hh signalling pathway (51,53).

Sheng *et al* (54) reported that bufalin was able to inhibit liver cancer cell EMT and ECM degradation by affecting the Gli1 and Gli3 expression of the Hh signalling pathway. Bufalin inhibited the expression of MMP2, MMP9, β-catenin and vascular endothelial growth factor in liver cancer cells and upregulated E-cadherin expression. Another study also demonstrated that bufalin was able to suppress cancer stem-like cells in gemcitabine-resistant pancreatic cancer via Hh signalling. Bufalin was also shown to inhibit the colony formation of pancreatic cancer cells and reduce tumourigenesis in nude mice. Western blotting and immunohistochemical results revealed that CD24 and epithelial-specific antigen levels were downregulated after treatment with bufalin. Bufalin was also shown to inhibit metastasis in nude mice injected with tumour cells via the tail vein. Moreover, Hh signalling was found to be suppressed in bufalintreated cells (22).

7. Other antimetastatic effects of bufalin

Bufalin inhibits NF-κB activation. NF-κB was first identified by Dr Ranjan Sen via its interaction with an 11-base pair sequence in the immunoglobulin light-chain enhancer in B cells (55). NF-κB plays an important role in immune response, as well as in cancer initiation and progression. When NF-κB is activated, preneoplastic and malignant cells exhibit increased anti-apoptotic ability. NF-κB is also implicated in tumour angiogenesis and invasiveness. Thus, NF-κB and its pathway are promising targets in anticancer therapy (56). It was reported that bufalin can inhibit hepatocellular carcinoma invasion and metastasis. Functional studies demonstrated that bufalin inhibited the expression of MMP2, MMP9 and PI3K and suppressed the phosphorylation of AKT, which was associated with a reduction in the level of NF-κB (42).

Bufalin modulates the activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signalling pathway. MAPKs are serine-threonine kinases that are involved in diverse biological activities, such as cell proliferation, differentiation, survival, death and transformation (57). The MAPK family includes ERK, p38 and c-Jun NH2-terminal kinase (JNK). Both extracellular and intracellular stimuli can promote the activation of MAPK pathways, such as peptide growth factors, cytokines, hormones, oxidative stress and endoplasmic reticulum stress (58). A number of studies have demonstrated that the abnormal expression of MAPK signalling pathways is important in tumour development. The ERK pathway also increases the expression of MMPs, promotes the degradation of ECM proteins, and enhances the invasion ability of cancer cells (59,60).

Hong *et al* (23) reported that bufalin attenuated the migratory and invasive abilities of bladder cancer cells. Following

treatment with bufalin, the expression of transepithelial electrical resistance increased, which promoted the expression of tight junctions. Bufalin also inhibits the expression of MMP2 and MMP9, while increasing the expression of metalloproteinase inhibitor. All these molecules are associated with the activation of the ERK pathway. Chueh *et al* (61) also found that bufalin inhibited the migration and invasion of human osteosarcoma U-2 OS cells and reduced the levels of MAPKs, such as JNK1/2 and ERK1/2.

Bufalin prevents cell migration by regulating the expression of miRNAs. MicroRNAs (miRNAs) are non-coding RNAs, ~22 nucleotides in length and highly conserved. miRNAs can regulate gene expression by binding to target sequences in mRNAs to induce mRNA degradation or suppress translation. miRNAs regulate various cellular processes, such as metastasis, proliferation and chemosensitivity. Studies have demonstrated that a number of malignant tumours have disrupted regulation of miRNAs that exert anticancer or tumour-promoting effects (62-65). miRNA profiling has become a diagnostic and prognostic marker as well as a therapeutic target for cancer (66). Qiu *et al* (67) demonstrated that bufalin can inhibit the migration of human colon cancer cells (HCT116) and human umbilical vein endothelial cells (HUVECs). They observed that miR-497 was upregulated in human colorectal cancer HCT116 cells treated with different concentrations of bufalin. In addition, bufalin was found to enhance the expression of the tumour suppressor miR-497 in nude mice.

In conclusion, bufalin is a potential polygenic and multitarget anticancer agent (Fig. 1) that can inhibit EMT by regulating the expression of TGF-β, PI3K/AKT, Hh and Wnt/β-catenin signalling pathways, thereby preventing metastasis. Bufalin also inhibits cancer metastasis by suppressing NF-κB activation and regulating the MAPK/ERK signalling pathway, as well as the expression of certain miRNAs. All these signalling pathways are interrelated; for example, bufalin modulates the PI3K/AKT signalling pathway, and PI3K/AKT activity in turn regulates the expression of NF-κB in hepatocellular carcinoma (42). However, the study of the anticancer and antimetastatic properties of bufalin is in its initial phases, and the underlying molecular mechanism remains unknown. Studies are currently limited to fundamental research, and several more studies are required before bufalin can be used in a clinical setting. However, with additional studies investigating the effect of bufalin on oncogenic cell signalling pathways or targets, it has the potential to become a drug for targeting cancer metastasis.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

JW and TC made substantial contributions to the conception and design of the study, and wrote the manuscript, JW, YX and Q-SZ performed literatures search regarding bufalin anticancer activity. The final version of the manuscript has been read and approved by all authors.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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